BIOLOGY OF REPRODUCTION **64,** 689–695 (2001)

Specific Staining of Sertoli Cell Nuclei and Evaluation of Sertoli Cell Number and Proliferative Activity in Meishan and White Composite Boars During the Neonatal Period[1]

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ABSTRACT

The positive relationship between Sertoli cell number and testicular size emphasizes the importance of determining factors involved in the regulation of the Sertoli cell population. Based on data from other species and indirect evidence in the boar, it is generally accepted that porcine Sertoli cells proliferate rapidly throughout the early postnatal period. However, direct evaluation of Sertoli cell number and the proliferative activity of Sertoli cells during the early postnatal period in boars have not been reported. Stereological enumeration of Sertoli cells is a labor-intensive process and would be greatly facilitated by a marker for these cells especially in the sexually mature male. Thus, the first objective of this study was to determine if expression of the transcription factor GATA-4 is an effective marker for fetal, postnatal, and adult Sertoli cells to facilitate enumeration procedures. The second objective was to evaluate the proliferative activity and growth of the Sertoli cell population in neonatal White Composite and Meishan boars, known to differ in mature testis size and Sertoli cell number, to determine the importance of this developmental period for the adult Sertoli cell population. GATA-4 was abundantly expressed by Sertoli cells throughout fetal and prepubertal stages of development and specifically stained both type A and B Sertoli cell nuclei in the sexually mature boar. Immunoreactivity was never observed in the germ cells regardless of their stage of development, illustrating that GATA-4 is a useful marker for both developing and adult Sertoli cells in the boar. Testicular size did not differ between breeds on Day 1 postpartum, but by 14 days postpartum White Composite boars had significantly larger testes compared to Meishan boars ($P < 0.001$). Similarly, Sertoli cell number did not differ between breeds at 1 day postpartum; however, at 14 days postpartum White Composite boars had a significantly larger Sertoli cell population compared to Meishan boars ($P < 0.05$). Surprisingly, despite having more Sertoli cells than Meishan boars at 14 days postpartum, the proportion of actively proliferating Sertoli cells in the White Composite boars was almost 50% less than the Meishan boars. This result illustrates that rapid rates of Sertoli cell proliferation probably occurred prior to 14 days postpartum in the White Composite boars. Collectively, these results illustrate that the relationship between testicular size and Sertoli cell number is manifested very early in the postnatal period for these two breeds. The substantial difference in the size of the Sertoli cell population and their proliferative activity between Meishan and White Composite boars during the early postnatal period emphasizes the importance of this early period for the establishment of the Sertoli cell population and subsequent adult testicular size.

early development, gamete biology, puberty, Sertoli cells, testis

INTRODUCTION

Sertoli cells play an important role in both testicular development and function, as they provide an environment that protects and nurtures the germ cells and assists their development into viable sperm. In addition, Sertoli cell number determines subsequent adult testicular size and capacity to produce sperm [1–3]. Sperm production in boars has become more economically important due to the increased demand for artificial insemination by swine producers. Technology that produces boars with larger testes would decrease production costs and simultaneously maximize the opportunities to use genetically superior sires. Thus, identifying and modifying factors that regulate Sertoli cell number may ultimately enhance reproductive efficiency in boars.

Sertoli cells can first be identified following male sexual differentiation at approximately 26 days postcoitus (dpc) in the pig [4]. During fetal life and the early postnatal period, seminiferous tubules contain only two types of cells: Sertoli cells and occasional large germ cells [5]. A marked preponderance of Sertoli cells within the testicular cords (tubules) is evident throughout fetal and early postnatal life until the onset of spermatogenesis at puberty. At this time, germ cells undergo extensive proliferation and the germ cell:Sertoli cell ratio reverses such that the proliferating germ cells far outnumber their somatic counterparts. In the sexually mature boar testis, two types of Sertoli cells can be identified at the electron microscope level. Type A Sertoli cells have fine structural characteristics similar to Sertoli cells observed in other species; i.e., a light staining nucleus, and contain numerous filaments and ribosomes, abundant smooth endoplasmic reticulum (SER), and typical junctions with adjacent Sertoli cells and germ cells [6]. These cells extend from the basement membrane to the lumen of the tubule and are routinely classified as Sertoli cells even at the light microscope level. Type B Sertoli cells are less numerous and are found only in close apposition to the basement membrane of the seminiferous epithelium. They are small cells with dark nuclei and limited cytoplasm containing SER and numerous filaments [6]. At the light microscope level, type B Sertoli cells are difficult to identify as they are similar in appearance to adjacent differentiating spermatogonia. Therefore, it is probable that studies evaluating Sertoli cells in the sexually mature boar at the light microscope level may evaluate only type A Sertoli cell nuclei. The functional significance of type B Sertoli cells is presently unknown. In addition, stereological eval-

[1]Mention of product names is necessary to report factually on available data; however, the U.S. Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the project to the exclusion of others that may also be suitable.

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Received: 1 August 2000.
First decision: 22 August 2000.
Accepted: 3 October 2000.

ISSN: 0006-3363. http://www.biolreprod.org

uation of Sertoli cells using light microscopy is a labor-intensive process [2, 7, 8], and therefore, a marker that specifically stains both types of Sertoli cell nuclei would greatly facilitate the speed and accuracy of stereological enumeration procedures.

During fetal and early postnatal life in mice, the transcription factor GATA-4, a zinc finger protein implicated in the regulation of gene expression and differentiation in a variety of tissues, is abundantly expressed in the nuclei of Sertoli cells but not germ cells [9], indicating its potential as a marker for Sertoli cells. The expression of GATA-4 in the porcine testis has not been characterized. Therefore, the objective of the first experiment was to determine the usefulness of GATA-4 as a marker for Sertoli cell nuclei in the developing and adult porcine testis.

At maturity, Meishan (MS) boars have lower testicular weights, fewer Sertoli cells per gram of testicular tissue, a greater percentage of interstitial structures, a lower percentage of seminiferous tubules and a reduced total daily sperm production compared to European breeds [7, 8, 10]. Based on data from other species and indirect evidence in the boar [11–14], it is generally accepted that porcine Sertoli cells proliferate rapidly throughout the early postnatal period. Both MS and conventional boars exhibit a major peak in serum follicle stimulating hormone at 1–2 wk postpartum [10] that may be associated with rapid proliferation of Sertoli cells. However, direct evaluation of Sertoli cell number and the proliferative activity of Sertoli cells during the early postnatal period in boars have not been reported. Therefore, the objective of the second experiment was to compare changes in testicular weight, Sertoli cell number, and their proliferative activity between MS and White Composite (WC) boars at 1 and 14 days postpartum (dpp) to determine whether this period is important for the establishment of the mature Sertoli cell population.

MATERIALS AND METHODS

Sample Collection and Histological Preparation

Experiment 1. Testes from fetal (30–40 and 105 dpc), prepubertal (1, 14, and 56 dpp), and postpubertal (240 and 420 dpp) WC boars, representing at least three litters per age (i.e., n = 3–6 animals per age group), were collected to determine the potential utilization of the transcription factor GATA-4 as a specific marker for Sertoli cells.

Experiment 2. Six MS and six WC boars, each from different litters, were castrated on 1 dpp, and full sibs of these boars castrated on 14 dpp, for evaluation of testicular morphological characteristics.

In both experiments, paired testes were trimmed of epididymides and excess connective tissue and weighed. Fetal testes were processed intact; Day 1 and 14 postpartum testes were cut longitudinally into two pieces, and 1-cm^3 parenchyma samples were taken from the older testes. Tissues were fixed overnight in 4% paraformaldehyde in PBS with gentle agitation. Each sample was washed in PBS (2 × 1 h), dehydrated through graded ethanol (70, 80, 95, 100%; 2 × 1 h each), cleared in xylene (2 × 1 h; Sigma, St. Louis, MO), infiltrated with paraffin (60°C; 4 × 1 h), and embedded in paraffin wax. Eight full-face longitudinal serial sections (5 μm) were made from the middle of one testis per animal. Approximately the same region of each testis was used. Cross-sections were dried overnight onto glass slides at 37°C and stored at room temperature until staining. Sections were subjected to immunohistochemistry as described below.

Immunohistochemistry Procedures

GATA-4 localization. The antibody used in the immunolocalization studies was an affinity-purified goat anti-mouse polyclonal antiserum (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) raised against a peptide mapping at the carboxy terminus of GATA-4 of mouse origin. This epitope is completely conserved between mouse and humans and 90% identical in pigs (AAF05950; representing the terminal 110 amino acids of porcine GATA-4). Sections were deparaffinized in Microclear (2 × 5 min; Micron Environmental Industries, Fairfax, VA) and rehydrated through graded ethanol (2 × 100%, 2 × 95%, 1 × 70%). Antigen retrieval was achieved by submerging the slides in 0.01 M sodium citrate, pH 6.0, and microwaving on high for 10 min. Sections were left submerged in 0.01 M sodium citrate, pH 6.0, for 10 min following this step. Incubating the slides in 3% hydrogen peroxide for 10 min quenched endogenous peroxidase activity. Immunolocalization of GATA-4 antibodies was achieved using the avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlingame, CA). Novared (Vector Labs.) was used as the chromagen. Briefly, sections were washed in Tris buffer (TB; 50 mM Tris, 1.5% NaCl, pH 7.6; 3 × 5 min) and incubated for 20 min in 1% normal rabbit serum to block nonspecific binding. Following a 1-h incubation with the GATA-4 primary antibody (1:200), sections were washed in TB (3 × 5 min) and incubated with the biotinylated secondary antibody for 40 min. Following further TB washes (3 × 5 min) and incubation with the ABC reagent for 40 min, immunoreactivity was visualized using Novared as the chromagen. Sections were rinsed thoroughly in water, lightly counterstained with hematoxylin, dehydrated, cleared in Microclear, and mounted using DPX mounting media (Fluka Biochemica, Steinheim, Germany). Slides were stored at room temperature until morphometric analysis.

Sections were also subjected to the same immunohistochemical procedure outlined above using the commercially available GATA-4 blocking peptide (Santa Cruz Biotechnology) in 10× excess of the primary antibody to confirm the specificity of the GATA-4 antibody. In addition, nonimmune serum or absence of the primary antibody was used to conclude that nonspecific binding was not problematic. Identification of GATA-4-positive nuclei as Sertoli cell nuclei was confirmed by the colocalization of Müllerian-inhibiting substance (unpublished observations).

Ki67 localization. Cross sections were prepared as above prior to the addition of the primary antibody. Immunolocalization of Ki67 antigen, a nonhistone nuclear protein found in all phases of the cell cycle except G0 [15], was achieved using the Ki67 Antigen Kit (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). Briefly, sections were incubated with the mouse monoclonal primary antibody for 2 h and washed in TB (3 × 5 min). Following a 40-min incubation with the biotinylated rabbit anti-mouse secondary antibody (1:500), sections were washed in TB (3 × 5 min) and incubated for 40 min in the ABC reagent. The immunoreactivity was visualized using Novared as the chromagen. Sections were washed, counterstained with hematoxylin, dehydrated, cleared, mounted, and stored as described above. Serial sections were also processed using nonimmune serum or absence of the primary antibodies to conclude that nonspecific binding was not problematic.

Stereological Evaluation of Testicular Composition

The 5-μm sections stained immunohistochemically for either GATA-4 or Ki67 were examined under brightfield microscopy and used to determine Sertoli cell number and the proportion of proliferating Sertoli cells, respectively. All measurements were made using computerized morphometric planimetry (Bioquant IV system; R&M Biometrics, Nashville, TN). Full cross sections from near the middle of each testis were used in these evaluations. Four areas randomly selected to represent each quadrant on each section were evaluated. The proportions (volume percentages) of the parenchyma composed of seminiferous tubules or interstitial tissue were determined at a magnification of 200×. Each area was projected onto a computer screen, and the tubular components of the parenchyma were measured by tracing outlines of whole seminiferous tubules using a computer mouse connected to a high-resolution digital pad. Thus, approximately 5×10^6 μm^2 was evaluated per testis (approximately 1.2×10^6 μm^2 evaluated per quadrant). At least 250 seminiferous tubules were measured at 200× per testis. For determination of nuclear size, four round tubules were selected within each quadrant, and Sertoli cell number, germ cell (prespermatogonia) number, and nuclear volumes within each tubule were determined at a magnification of 1000×. For each cell type, the outline (perimeter) of every nucleus within each of the four round tubules per quadrant was traced. At least 600 Sertoli nuclei and 40 germ cell nuclei were measured at 1000× per testis in each age group.

The average volume of each nucleus was calculated using the formula for a prolate sphere ($4/3\pi ab^2$; where a = long radius and b = short radius derived from the Bioquant morphometry). To adjust for capping effects, nuclear volumes were corrected using Abercrombie's formula ($4/\pi$) [16]. No correction factor for shrinkage was applied as the samples used for comparison were fixed and subsequently processed using identical conditions, and thus the extent of shrinkage was assumed to be equal for each group. Area measurements from the Bioquant System were used to calculate the volume percentage of the testis occupied by seminiferous tubules and the volume percentage of the testis occupied by interstitial tissue including Leydig cells (i.e., volume densities). To determine the total number of Sertoli cells and germ cells on a per organ basis, the formula of Wreford [17] was used, where the total number of each type of nuclei per testis was the product of testis volume and volume density of nuclei in the testis (i.e., total mass of each type of nuclei per testis) divided by the mean nuclear volume. The number of cells per gram of tubule was calculated by dividing the total nuclei per organ by the total tubular mass per testis (i.e., product of testis volume and volume density of tubules in the testis).

The number of Sertoli cells undergoing proliferation was determined by repeating the above procedure at 1000× for those Sertoli nuclei labeled with the Ki67 antigen. Given the limited number of Sertoli cells per tubule undergoing proliferation, as compared to the total population, a total of eight round tubules per quadrant per animal was evaluated. Thus, at least 1200 Sertoli nuclei were evaluated for Ki67-labeled proliferation per testis at 1000×. The number of germ cells (i.e., prespermatogonia) undergoing proliferation was extremely low at these early postnatal stages of testicular development; thus, proliferative activity in germ cells was not enumerated.

Statistical Analysis

Differences between breeds in all the components estimated were tested using a mixed model procedure [18]. The model included the fixed effects of breed and quadrant and the random effects of boar. All testicular composition data were transformed ($\log_{10}$) prior to statistical evaluation to adjust for heterogeneity of variances. For presentation, these data are reported as arithmetic means and standard errors.

RESULTS

Experiment 1

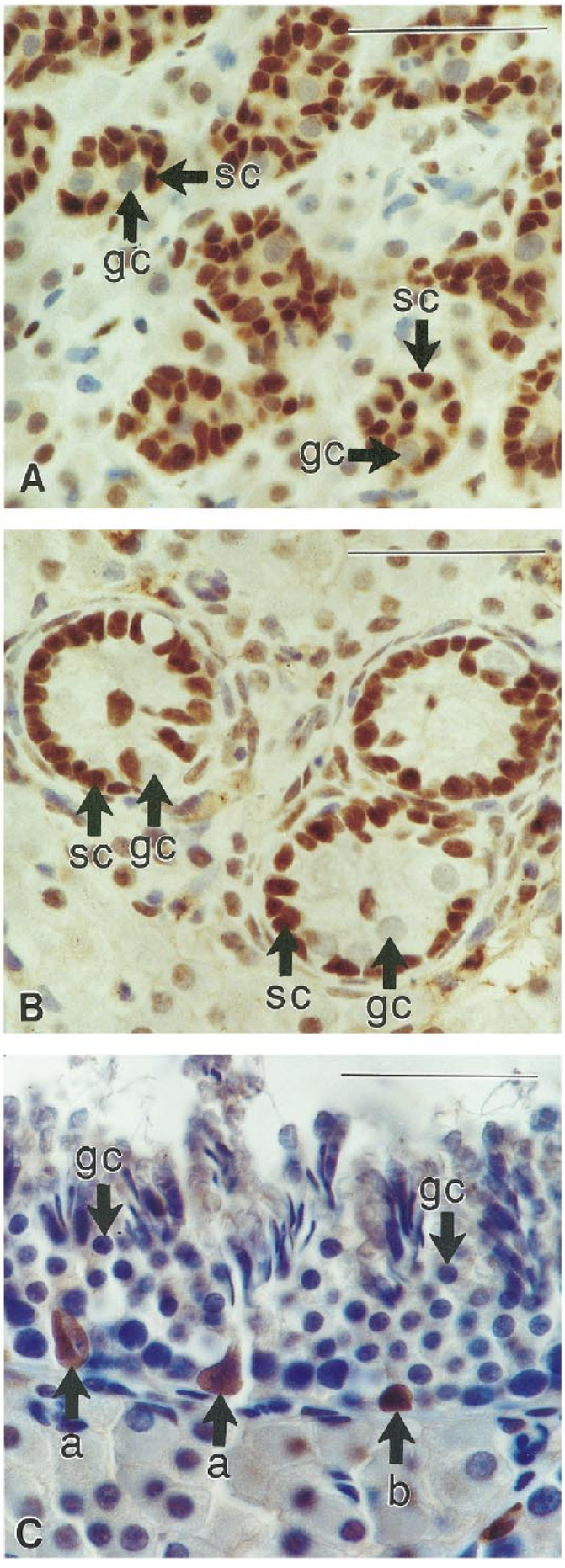
GATA-4 as a marker for Sertoli cells. Within the seminiferous tubules, GATA-4 was abundantly expressed within the nuclei of every Sertoli cell present. Expression of GATA-4 within Sertoli cells was apparent during fetal (30–40 and 105 dpc), prepubertal (1, 14, and 56 dpp) and postpubertal (240 and 420 dpp) stages of development. More importantly, GATA-4 specifically stained all Sertoli cell nuclei inside the tubules in the sexually mature boar. Representative staining of GATA-4 in Sertoli cell nuclei of fetal (40 dpc), prepubertal (14 dpp), and postpubertal (420 dpp) boar testes are shown in Figure 1. GATA-4 was not expressed in germ cell nuclei at any stage evaluated. In the interstitium, outside the tubules, GATA-4 was expressed at low levels in Leydig cell nuclei and in some peritubular myoid cells, but staining was never observed in endothelial cells at any age.

In postpubertal boars, GATA-4 staining was never observed in germ cell nuclei irrespective of their stage of maturity. In contrast, GATA-4 staining was present in all Sertoli cell nuclei within the tubules. Of the GATA-4-positive cells, differential interference microscopic examination at 1000× magnification confirmed the identity of two types of Sertoli cells based on morphological characteristics. Type A Sertoli cells (Fig. 1C) displayed large irregular nuclei containing distinct nucleoli and had extensive cytoplasmic processes extending from the basement membrane to the lumen of the tubule, typical of functional Sertoli cells. Type B Sertoli cells (Fig. 1C) were small cells that had limited cytoplasm and resembled adjacent primary spermatogonia but contained distinct nucleoli characteristics of Sertoli cells and were always located on the basement membrane. Based on cellular, nuclear, and nucleolar characteristics, only Sertoli cells exhibited GATA-4 staining, inside seminiferous tubules.

Experiment 2

Breed effects on testis weight and composition in the neonate. No difference in testicular weight at 1 dpp was observed between MS and WC boars. However, by 14 dpp, testicular weight was substantially greater in WC boars compared to MS neonatal boars (Fig. 2).

Within breed, the proportion of the testis occupied by tubules did not change between 1 and 14 dpp (Table 1). However, MS boars had a twofold greater proportion of the testis occupied by tubules at both ages than did WC boars, with a concomitant decrease in the proportion occupied by interstitial tissue (Table 1). Despite a difference in the proportion of the testis occupied by tubules, total tubular mass was not different between the two breeds within either age group (Table 1). However, age had a significant effect, and tubular mass increased four- to sevenfold between 1 and 14 dpp in both breeds.



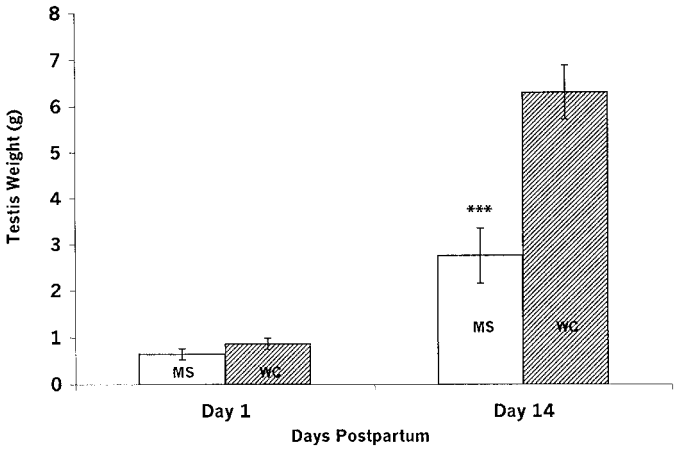


FIG. 2. Paired testicular weights of Meishan (MS) and White Composite (WC) neonatal boars on 1 and 14 days postpartum. Data are means ± SEM. ***$P < 0.001$; breed difference within day.

The total number of Sertoli cells per paired testes was not different between breeds at 1 dpp (Fig. 3). Although the absolute number of Sertoli cells increased two- to fourfold in each breed between 1 and 14 dpp, MS boars had fewer ($P < 0.05$) Sertoli cells than WC boars by 14 dpp. Interestingly, the number of Sertoli cells per gram of tubule was lower in the MS than the WC neonatal boars at both ages studied, but Sertoli cell nuclear volume was larger ($P < 0.05$) in MS neonates at both ages (Table 1). However, the number of Sertoli cells per gram of testicular tissue was greater in MS than in WC boars, reflecting the greater volume percentage of tubules present in the MS boars (Table 1).

Based on the presence of Ki67 antigen, intriguing differences were observed in the number of proliferating Sertoli cells within the developing testis. At both 1 and 14 dpp, MS boars had a twofold greater ($P < 0.05$) proportion of the Sertoli cell population undergoing proliferation than did WC boars (Fig. 4). The proportion of proliferating Sertoli cells also increased with advancing age in both breeds. The number of proliferating Sertoli cells per gram of tubule was consistently greater in MS than WC boars at both neonatal ages studied (Day 1: 61.03 ± 12.49 vs. 41.73 ± 5.99, $P < 0.10$; Day 14: 49.82 ± 7.54 vs. 33.49 ± 4.74, $P < 0.05$; 10^7).

The germ cell population size and individual germ cell nuclear volume was greater ($P < 0.05$) in the MS boars than in WC boars at 1 dpp. However, these differences had disappeared by 14 dpp (Table 1). When expressed as a ratio, the number of germ cells per Sertoli cell was also greater in the MS than WC boars ($P < 0.01$) at 1 dpp, but no difference was apparent by 14 dpp.

FIG. 1. Immunolocalization of GATA-4 in Sertoli cell (sc) nuclei in fetal (40 days postcoitus; **A**), prepubertal (14 days postpartum; **B**), and both type A (a) and type B (b) Sertoli cell nuclei in postpubertal (420 days postpartum; **C**) White Composite boars. Positive staining is depicted by red color. Sections shown for each age group are counterstained blue with hematoxylin to illustrate unstained germ cell (gc) nuclei within the seminiferous tubules. Note in **C** the numerous germ cell nuclei at various stages of development. Magnification bar on each micrograph represents 50 μm.

TABLE 1. Characteristics of testicular structures between Meishan (MS) and White Composite (WC) neonatal boars at 1 and 14 days postpartum; data are arithmetic means ± SEM.

	Day 1		Day 14	
	MS	WC	MS	WC
Volume % seminiferous tubules	31.0 ± 0.03	15.7 ± 0.02*	29.1 ± 0.03	15.1 ± 0.01*
Volume % interstitial tissue	69.0 ± 0.03	84.3 ± 0.02*	70.9 ± 0.03	84.9 ± 0.01*
Tubular mass (mg/paired testes)	191.1 ± 38.6	127.1 ± 8.89	776.1 ± 108.4	932.9 ± 80.4
No. Sertoli cells (10^7/g tubule)	326.8 ± 18.9	421.7 ± 35.9‡	162.9 ± 39.1	217.9 ± 33.5§
No. Sertoli cells (10^7/g testis)	99.6 ± 10.2	64.7 ± 6.7†	44.8 ± 8.9	32.2 ± 4.4‡
Sertoli cell nuclear volume (μm^3)	161.2 ± 10.9	123.4 ± 9.9‡	283.2 ± 24.5	200.3 ± 15.1†
Total no. germ cells (10^6/paired testes)	20.6 ± 4.0	13.7 ± 2.1‡	39.9 ± 5.6	53.2 ± 9.5
Total no. germ cells (10^6/g tubule)	117.3 ± 16.1	108.7 ± 15.6	54.4 ± 7.1	57.7 ± 9.4
Germ cell nuclear volume (μm^3)	1864.9 ± 182.1	1457.8 ± 147.6‡	3488.9 ± 236.9	3217.2 ± 270.1

* $P < 0.001$; breed difference within day.
† $P < 0.01$; breed difference within day.
‡ $P < 0.05$; breed difference within day.
§ $P < 0.10$; breed difference within day.

DISCUSSION

Experiment 1

In the present study, GATA-4 specifically stained all Sertoli cell nuclei located within the seminiferous tubules, and some interstitial cells during fetal and early postnatal development. Expression was always nuclear, consistent with its role as a transcription factor. Inside the seminiferous tubules, nuclear staining was very intense and the boundaries of the nuclei were clearly delineated enabling clear definition between adjacent Sertoli cells. This is particularly important for the enumeration of Sertoli cells prior to puberty when most Sertoli cell nuclei are tightly packed along the basement membrane of the tubules. GATA-4 immunoreactivity was never observed in the large round primordial germ cells nor prespermatogonia at any stage studied. Careful examination of the GATA-4 immunoreactive cells within the tubules in the sexually mature boar revealed that GATA-4 specifically stained both types of Sertoli cell nuclei. Of the GATA-4-positive nuclei, type A Sertoli cells were easily identified by their extensive cytoplasmic processes and irregular nuclei. In addition to these easily recognizable Sertoli cells, another group of cells was immunoreactive for GATA-4. These type B Sertoli cells had small round nuclei containing distinct nucleoli, substantially less cytoplasm, and were always located very near the basement membrane of the seminiferous epithelium as described by Chevalier [6]. Type B Sertoli cells are not easily recognizable as Sertoli cells using light microscopy, and it is probable that their nuclei are not classified as Sertoli cell nuclei using classical evaluation procedures. Therefore, the specific GATA-4 staining in both types of Sertoli cell nuclei enables their rapid identification using light microscopy and greatly increases the speed and accuracy of enumeration procedures in the sexually mature boar. Based on the abundant and persistent expression exclusively within the nuclei of Sertoli cells throughout fetal and postnatal life, we conclude that GATA-4 is a reliable marker for Sertoli cells in the developing and adult porcine testis.

The expression of GATA-4 in Sertoli cells throughout the fetal and postnatal developmental period in the boar is in contrast to the expression pattern observed in the mouse. In mice, GATA-4 expression is rapidly down-regulated in Sertoli cells at the onset of spermatogenesis [9], whereas in the pig, GATA-4 continues to be expressed beyond puberty and into maturity. This is likely to be due to an intrinsic species difference rather than a reflection of the antibody used to detect GATA-4, as the antibody used in both studies

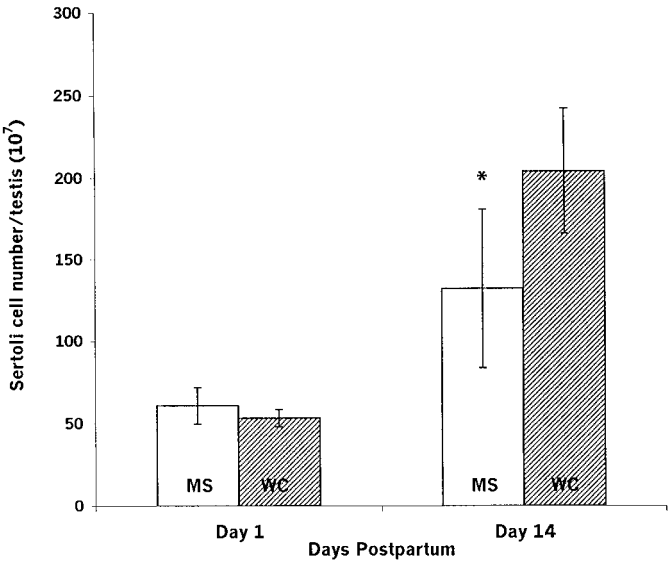


FIG. 3. Estimated total Sertoli cell number (10^7) within the tubular compartment of the testis for Meishan (MS) and White Composite (WC) neonatal boars on Day 1 and 14 postpartum. Data are arithmetic means ± SEM. *$P < 0.05$; breed difference within day.

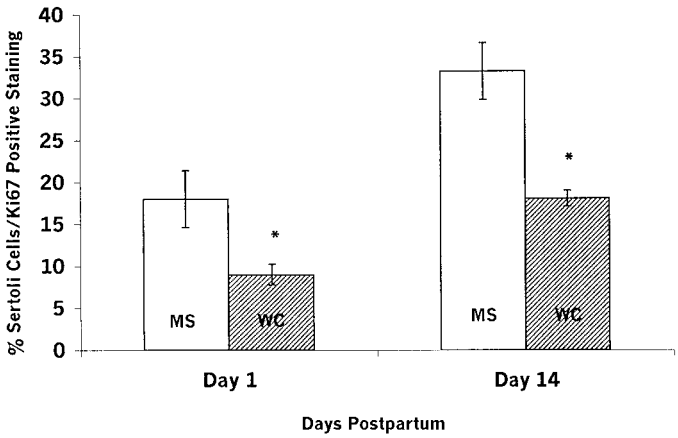


FIG. 4. The percentage of the total Sertoli cell population undergoing proliferation in Meishan (MS) and White Composite (WC) neonatal boars at 1 and 14 days postpartum. Data are arithmetic means ± SEM. *$P < 0.05$; breed difference within day.

was identical (polyclonal goat anti-mouse C-20; Santa Cruz Biotechnology). However, antigen retrieval was performed in the present study but was not used in the mouse studies. Thus, the sensitivity of the staining procedure employed in this study may be greater and could account for some of the staining differences observed between species.

Experiment 2

At maturity, MS boars have considerably smaller testes coupled with a reduction in Sertoli cell number compared to WC boars [7, 8, 10], offering a unique model to study factors controlling Sertoli cell number and thus testis size. The early postnatal period, a time of elevated gonadotrophin secretion, has been highlighted as an important period for establishing the mature Sertoli cell population size [12–14, 19]. However, direct evaluation of Sertoli cell number and their proliferative activity during the early postnatal period in boars have not been reported. Therefore, the second objective of the present study was to determine the importance of the early postnatal period for the establishment of the Sertoli cell population, and thus testicular size, in MS and WC boars.

Testicular size did not differ between the breeds at 1 dpp. However, by 14 dpp there was a twofold difference in testicular size in favor of WC neonatal boars. The magnitude of this difference in testicular size at 14 dpp is similar to the difference observed between these two breeds at maturity [7, 8, 10]. Consistent with testicular size, the Sertoli cell population size did not differ between breeds at 1 dpp. However, the greater testicular weight in WC boars evident at 14 dpp was associated with a concomitant increase in Sertoli cell number but not a significant increase in tubular mass at this stage of development. This result illustrates that the relationship between testicular size and Sertoli cell number is manifested very early in the postnatal period, further emphasizing the influence of this early period for mature testicular size.

The testis is comprised of two major compartments: seminiferous tubules that contain the machinery to facilitate spermatogenesis, and the interstitial compartment that houses the steroidogenic machinery of the testis. While these two compartments work together to support spermatogenesis, they are two functionally distinct compartments. Despite a similar mass of seminiferous tubules, the volume percentage of the testis occupied by tubules was twofold greater in MS than in WC neonates. This reflects a smaller volume percentage of the testis occupied by interstitial space. This relationship is reversed in sexually mature MS and WC boars, i.e., interstitial space comprises a larger proportion of the testis in MS than WC boars [10], illustrating that these two compartments of the testis develop at different rates in these two breeds.

There are two classical methods to present Sertoli cell enumeration data to illustrate biological changes/effects within the testis. The first method represents Sertoli cell number per gram of seminiferous tubule. This represents the number of Sertoli cells per unit of spermatogenic tissue (seminiferous tubule) present in the whole testis and does not account for the contribution of the interstitial compartment to testicular mass. In the present study, despite a similar mass of seminiferous tubules tissue between breeds, WC neonates had more Sertoli cells per gram of seminiferous tubule compared to MS neonates, which may simply reflect the substantially larger Sertoli cell nuclear volume present in the MS boars. This was confirmed by direct measurement of Sertoli cell nuclear size. This indirect approach has previously been used to estimate changes in the Sertoli cell population during late fetal and postnatal life [20–22]. However, the present study illustrates that making the assumption that a similar mass of tubules represents a similar number of Sertoli cells may not be valid in some biological systems such as breed comparisons. Therefore, it is advisable to determine Sertoli cell number to lead to biologically accurate conclusions.

The second method represents Sertoli cell number per gram of testicular tissue (seminiferous tubule plus interstitial space). If the proportion of the testis occupied by interstitial space and tubular tissue is similar for both groups being compared, this method yields a similar biological (but not numerical) result to the first method. However, in situations when the volume percentage of the testis occupied by interstitial tissue or seminiferous tubules is not similar, presenting data in this way is biologically misleading. The present study demonstrates how representation of data in only one way could yield misleading results and lead to biologically inaccurate conclusions. When the number of Sertoli cells is expressed per gram of testicular mass, MS boars appear to have a greater number of Sertoli cells per gram of testicular tissue compared to WC neonates. However, the opposite relationship is true when Sertoli cell number is expressed per gram of seminiferous tubule. This discrepancy results from breed differences in the volume of the testis occupied by interstitial tissue and from the larger Sertoli nuclear volume in MS boars. We have presented the data in both ways in Table 1 to illustrate this point.

Sertoli cell mitotic activity is maximal during late fetal and the early postnatal period in the rat [23, 24], mouse [25], and rabbit [26]. Mitotic activity steadily declines during the postnatal period and ceases entirely following the onset of spermatogenesis [27]. In boars, the mitotic activity of Sertoli cells during the late fetal period is unknown; however, it has been implied that mitotic indices are high during the early postnatal period, following which they decline [11, 13, 14]. The apparent high rate of proliferation during this early postnatal period affirms its importance for determining the size of the mature Sertoli cell population.

In contrast to rodents where mitotic activity is maximal in late fetal and early postnatal life [23–25], the proportion of porcine Sertoli cells undergoing proliferation increased from 1 to 14 dpp. This indicates that the maximal rate of proliferation occurs after birth, either before or after 14 dpp consistent with observations in the rabbit [26]. Surprisingly, despite WC boars having more Sertoli cells than MS boars at 14 dpp, the proportion of Sertoli cells undergoing proliferation was almost 50% less than MS boars. This indicates that the proliferative activity of Sertoli cells differs between these two breeds during this early postnatal period. To enable the substantial increase in the number of Sertoli cells in the WC boars during this early postnatal period, the Sertoli cells must have undergone rapid proliferation between 1 and 14 dpp, highlighting the importance of this period for the establishment of the Sertoli cell population in the boar. The large increase in Sertoli cell number in WC versus MS boars during the first 2 wk of life indicates that factors controlling Sertoli cell proliferation act during this early postnatal period (or earlier) to regulate the growth of the Sertoli cell population. More frequent sampling during this early postnatal period is required to determine the changes in proliferation rate associated with increasing Sertoli cell population size.

In conclusion, the relationship between testis size and

Sertoli cell number in the boar is established early in the postnatal period. The substantial differences in testicular composition and Sertoli cell proliferative activity between MS and WC boars during this early postnatal period emphasize that the rapid changes occurring during early life have important implications for mature testicular size and reproductive efficiency in the boar.

ACKNOWLEDGMENTS

The authors thank Susan Hassler and Alan Kruger for their skillful technical assistance.

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